AFRL-RH-WP-TR-2013-0003

CORRELATION BETWEEN IRON AND α AND π GLUTATHIONE-S-TRANSFERASE LEVELS IN HUMANS

Camilla A. Mauzy
Nathan H. Johnson
Jason J. Jacobsen
Adam G. Quade
Jeremiah N. Betz
Molecular Bioeffects Branch
Bioeffects Division
Wright-Patterson AFB OH 45433-5707

Jeanette S. Frey
Amanda Hanes
Henry M. Jackson Foundation
For the Advancement of Military Medicine
Wright-Patterson AFB OH 45433-5707

David Kaziska
Air Force Institute of Technology
Wright-Patterson AFB OH 45433-7765

September 2012

DISTRIBUTION A.
Approved for public release; distribution unlimited.
Clearance# 88-ABW-2013-0726, 2-14-13.

Air Force Research Laboratory
711th Human Performance Wing
Human Effectiveness Directorate
Bioeffects Division
Molecular Bioeffects Branch
Wright-Patterson AFB OH 45433-5707

NOTICE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation; or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

This report was cleared for public release by the 88th Air Base Wing Public Affairs Office and is available to the general public, including foreign nationals. Copies may be obtained from the Defense Technical Information Center (DTIC) (http://www.dtic.mil).

AFRL-RH-WP-TR-2013-0003

THIS REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION IN ACCORDANCE WITH ASSIGNED DISTRIBUTION STATEMENT.

David R. Mattie

DAVID MATTIE, Work Unit Manager
Molecular Bioeffects Branch
Human Effectiveness Directorate
711th Human Performance Wing

G D Polhamus

GARRETT D. POLHAMUS, DR-IV, DAF
Chief, Bioeffects Division
Human Effectiveness Directorate
711th Human Performance Wing

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government's approval or disapproval of its ideas or findings.

REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
27-07-2012	Interim	May 2008 to Sept 2012

4. TITLE AND SUBTITLE
Correlation Between Iron Parameters and α and π Glutathione-S-transferase Levels in Humans.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
NA

5c. PROGRAM ELEMENT NUMBER
62202F

6. AUTHOR(S)
Camilla A. Mauzy, Nathan H. Johnson, Jason J. Jacobsen, Adam G. Quade, Jeremiah N. Betz, Jeanette S. Frey, Amanda Hanes, and David Kaziska

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER
OAFWP001

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
711 HPW/RHDJ, 2729 R Street, Wright-Patterson AFB OH 45433-5707

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Materiel Command
Air Force Research Laboratory
711th Human Performance Wing, Human Effectiveness Directorate
Bioeffects Division, Molecular Bioeffects Branch
Wright-Patterson AFB OH 45433-5707

10. SPONSOR/MONITOR'S ACRONYM(S)
711th HPW/RHDJ

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-RH-WP-TR-2013-0003

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES
DRAFT 88ABW/PA cleared 2-14-13, 88ABW-2013-0726

14. ABSTRACT Correlations were examined in humans of α and π glutathione-S-transferase s (serum and plasma, respectively) and blood levels of iron, transferrin, ferritin, soluble transferrin receptor (sTfR) as well as Total Iron-binding Capacity (TIBC), and percent Transferrin saturation (%TS). Data analysis of ungrouped (unbinned) data indicated significant gender differences in the blood levels of iron, TIBC, ferritin, and %TS, with none seen with transferrin, sTfR, or the GSTs. Regression analysis of ungrouped data with αGST as a response variable identified the interaction between age and iron as well as between age and percent iron saturation (%ISAT) as significant predictors of αGST. Regression analysis using ungrouped πGST data, only resulted in a quadratic model with iron as the only significant predictor ($R^2 = 6.9\%$). Using binned data based on published clinical levels, it was seen that elevated levels of iron and sTfR are strong predictors of elevated αGST levels. These associations were not seen using binned normal αGST levels. Unlike regression analyses of the ungrouped data, regression analyses of binned πGST data indicated significant associations (elevated iron, normal transferrin) although not as strong as those seen using αGST data.

15. SUBJECT TERMS
Glutathione-S-transferase , iron, transferrin, GST, human, sTfR, TIBC, ISAT, pi GST, alpha GST

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					Camilla A. Mauzy
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	SAR	39	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. 239.18

THIS PAGE INTENTIONALLY LEFT BLANK.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

PREFACE

This research was accomplished by a research group in the 711th Human Performance Wing, the Human Effectiveness Directorate under the Molecular Bioeffects Branch (RHDJ) within the Bioeffects (RHD) Division. Prior to 1 October 2011, this Branch was called the Applied Biotechnology Branch (RHPB) and previous reports and approvals on this project are listed under RHPB. This technical report was written for AFRL Work Unit OAFWP001, and is a follow-on study on the work reported in AFRL-HE-WP-TP-2006-0021.

This study was reviewed by the AFRL IRB which determined that it did not meet criteria for human subject research under the definition in 32 CFR 219.102(f). As the samples were obtained in the course of routine patient care, there was no interaction with humans for the purpose of this research, and the samples were de-identified.

ACKNOWLEDGEMENTS

The authors would like to thank Morgan Jacobsen and MSgt Christine Luther of the 88 DTS/SGQC for their help in obtaining the matched serum/plasma samples used in this study. The authors would also like to thank Drs. Victor Chan, Paul Eden, and Eric Holwitt for technical reviews and discussions.

1.0 SUMMARY

We have identified a number of potential biomarkers indicative of low level liver damage in response to uncharacterized toxin exposures. To conduct pre-validation and validation studies on these markers, blood and/or urine must be obtained in a human cohort with known sub-clinical hepatic damage. The acquisition of such samples is problematic – low level liver damage usually does not present primary or secondary effects that can be used a screen for potential subjects. In order to develop a non-invasive test method for such sample collections, we examined the levels of possible mild to increased liver damage in heterozygote hemochromatosis (HFE) samples as indicated by levels of the known hepatic biomarkers α and π glutathione-S-transferase s (GST) in the blood. HFE mutations can lead to liver damage caused by hepatocellular iron overload. As part of this research effort, correlations were examined using α and π glutathione-S-transferases (serum and plasma, respectively) and blood levels of iron, transferrin, ferritin, soluble transferrin receptor (sTfR) as well as Total Iron-binding Capacity (TIBC), and percent Transferrin saturation (%TS). A total of 90 matched samples (48 male, 42 female) of serum and plasma were obtained and tested for concentrations of all components. Data analysis of ungrouped (unbinned) data indicated significant gender differences in the blood levels of iron, TIBC, ferritin, and %TS, with none seen with transferrin, sTfR, or the GSTs. Regression analysis of ungrouped data with αGST as a response variable identified the interaction between age and iron as well as between age and percent iron saturation (%ISAT) as significant predictors of αGST. Regression analysis using ungrouped πGST data, only resulted in a quadratic model with iron as the only significant predictor (R^2 = 6.9%). Using binned data based on published clinical levels, it was determined that elevated levels of iron and sTfR are strong predictors of finding elevated αGST levels. These associations were not observed using binned normal αGST levels. Unlike regression analyses of the ungrouped data, regression analyses of binned πGST data indicated significant associations (elevated iron, normal transferrin) although not as strong as those seen using αGST data.

Keywords: glutathione-S-transferase, GST, pi GST, alpha GST, iron, transferrin, ferritin, transferrin receptor, liver damage, biomarker, serum, plasma, blood, biomarker

2.0 INTRODUCTION

2.1 Glutathione S-Transferases

2.1.1 Activity. The large protein family of glutathione S-transferases (GSTs) consists of constitutively expressed multi-role enzymes. These enzymes conjugate glutathione to a diverse array of both endogenous and exogenous xenobiotic compounds, a critical role in cellular detoxification (**Figure 1**).[1,2] GST enzymes catalyze a nucleophilic attack by gluthathione to electrophilic xenobiotics by activating a sulfhydryl group on glutathione. This reaction effectively eliminates reactivity seen in the 'free' xenobiotic thus providing a de-toxification mechanism within the cell. GSTs are also thought to be critical for removal of lipid peroxide products produced during oxidative stress.[3]

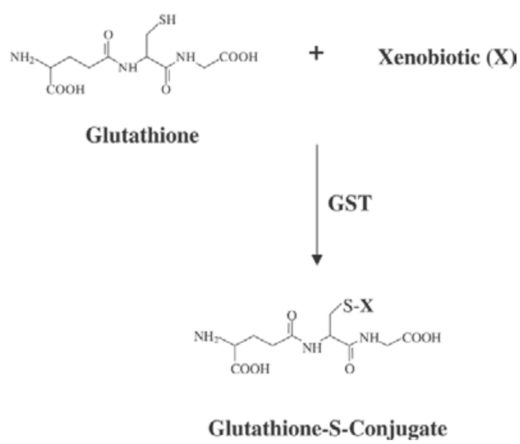

Figure 1. The role of GSTs in detoxification via conjugation with glutathione. Image taken from Townsend *et al*. 2003[4].

2.1.2. Subfamilies and expression. Mammalian cytosolic GSTs exist in several subfamilies (Alpha, Mu, Pi) and are classified based on primary/tertiary/quaternary structure as well as their kinetic properties.[5] Most GST species are expressed in multiple organs.[6] It has been shown that πGST is primarily located within the liver intrahepatic bile duct cells, whereas αGST is distributed throughout the liver with the highest concentration in hepatocytes.[7] By testing α or π GST levels in the serum or plasma, respectively, it has been shown that elevated levels of these proteins are indicative of site-specific hepatic injury.[8] Therefore, hepatic injury can monitored, at a fairly sensitive level, by determining α and/or π GST concentrations in the blood.

2.2 Cellular Iron Status in Humans

As there are no forms of the passive excretion of iron, its cellular levels are especially sensitive to perturbations. Normally, duodenal crypt cells determine the organism's need for iron and these adsorption requirements are "set" into maturing absorptive enterocytes. Once absorbed through the gut villi on the enterocytes, the divalent metal transporter 1 (DMT1) protein transfers the iron into the cell using a proton-coupled reaction.[9,10] For storage, iron is primarily sequestered in the ubiquitous globular protein complexes, ferritin[11] or hemosiderin.[12] αGSTs also can sequester forms of iron inside the cell via a nitric oxide-mediated reaction.[13]

Changes to iron uptake or metabolism can play a key role in mammalian health, with numerous disease states affiliated with altered iron homeostasis. There are many effectors of cellular iron concentration such as diet, malabsorption, *Helicobacter pylori* infection, drug interference, and hemorrhage.[14] Variants of hepcidin, considered the main regulator of iron homeostasis, as well as its regulatory proteins have been shown to change iron absorption.[15] Hereditary hemochromatosis type 1 is another well characterized disease of iron absorption. In this ailment, genetic mutations significantly alter iron levels in the blood by effecting either the concentration or the activity of key proteins involved in maintenance of iron status. One of these effectors, the hemochromatosis (HFE) gene, encodes a major histocompatibility complex protein. The HFE protein forms a heterodimer with β2-microblobulin, and this complex is embedded in the membrane surface of several types of cells, including duodenal crypt cells and macrophages. Several well characterized mutations in this gene have been shown to increase iron levels.[16] Of known HFE mutations, the cysteine to tyrosine change at position 282 (C282Y)[17] and possibly a histidine to aspartic acid at position 63 (H63D)[18] may alter iron absorption or protein concentration to initiate iron overload.[19] Iron overload in the blood can cause excess toxic iron deposition in key organs, namely the liver and heart, as well as lead to bone loss.[20] High levels of iron deposition in these tissues lead to damage due to the catalytic nature of soluble ferrous iron to react with soluble oxygen to produce oxygen free radicals and further down-stream reactive oxygen species (ROS).[21] Therefore an organism which demonstrates iron overload can, among other things, exhibit hepatic damage ranging from low level (pre-clinical) to massive organ failure.[22] Hemochromatosis is usually initially diagnosed by the use of blood tests examining the levels of serum transferrin saturation as well as serum ferritin. The saturation levels of transferrin are indicative of the amount of iron which is bound to transferrin in the blood. The ferritin tests the amount of iron stored in the liver. If suspected, secondary genetic tests can confirm the presence of the HFE mutations.

2.3 Modeling Human Sub-Clinical Hepatic Damage

Evaluation and validation of new biomarkers indicative of sub-clinical hepatic damage is problematic in that current clinical assays for hepatic damage are not sensitive enough to allow detection of low level damage. Past studies[23] have examined the effects of HFE gene mutations[24,25] on the development of low-level liver damage. Causative genetic mutations in the HFE gene were examined to see if the presence of the mutations correlated with low-level liver damage as indicated by elevated levels of serum αGST. It was hoped that successful correlation of the mutation with sub-clinical liver damage would allow the use of HFE genetic testing as a non-invasive method for collecting blood samples from persons with undetected liver damage. The identification of such a test model for sample collection is important in the development and validation of blood-based biomarkers capable of detecting early hepatic damage due to toxin exposures.[26] The conclusions of this study indicated that no statistically significant differences were seen in serum αGST levels in individuals heterozygous for the deleterious HFE H63D or C282Y mutations. Indeed, affected individuals seemed to have lower, not higher, αGST levels. However, there were several drawbacks to the study conducted. As the samples obtained were pre-existing clinical samples, there was concern that the sample quality may have been suboptimal for αGST enzyme-linked immunosorbant assay (ELISA) testing due to the length of time between sample draw and αGST analysis. The sample size also did not exhibit adequate populations of heterozyotes to make a statistically significant argument for the association of GST levels (and presumed hepatic damage) with the two mutations. In addition, only αGST was examined as only serum samples were collected. A πGST analysis using existing test assays requires plasma.

2.4 Correlation of Iron, αGST and πGST Levels in the Blood

An investigation of blood iron and GSTs may give insight into the use of GSTs as indicators of low level liver tissue damage as well as an understanding of their relationship to iron regulation. The purpose of the follow-on work was to examine the relationship between α and π glutathione-S-transferase and clinical iron levels. Therefore a second study was investigated to examine the possible correlations between αGST, πGST, iron and iron regulatory elements (transferrin, transferrin receptor, ferritin) using human matched serum/plasma samples. The previous study generated data from a total of 52 samples displaying heterozygote frequencies of 9.9% C282Y and 20.8 % H63D. We felt that, with these distributions, it would be difficult to collect enough matched samples to generate adequate statistical strength. Therefore in the new study, HFE mutations were not analyzed and only possible GST/iron associations examined. The 88th Medical group personnel selected matched serum/plasma samples from pre-existing clinical samples, and efforts were made to insure that the samples were received at RHDJ within the limits of assay time.

3. MATERIALS AND METHODS

3.1 Sample Collection

This study utilized pre-existing human serum and plasma samples drawn at WPAFB Medical center for other clinical analyses. Once the pre-existing samples were determined to be of no further utility to the Medical Center, matched serum/plasma samples were released to RHDJ. A total of 90 matched serum and plasma samples were collected in either SST or clot tubes without additive. The samples were stored at -80° C after transfer to RHDJ. Researchers did not have access to medical history data, only age/gender of each sample. The limited demographic information is presented in **Table 1**.

Table 1: Gender and Average Age of Serum Samples.

	Sample Number	**Average Age in Years** (± standard deviation)
Male	48	34.3 (11.3)
Female	42	33.2 (12.1)
Total	90	33.8 (11.6)

3.2 Enzyme-Linked Immunosorbent Assay (ELISA) Testing

3.2.1 αGST ELISA. Enzyme-linked immunosorbent assays were performed as described in the Biotrin High Sensitivity Alpha GST EIA kit protocol. First, serum samples were diluted 1:10 with wash solution (phosphate buffered saline with Tween 20). A total of 100 μL of diluted sample was added to the wells of 96-well microtiter plates coated with IgG directed against αGST and then incubated at room temperature (20-25^{o} C) for 60 min with uniform shaking. Plates were washed six times with wash solution. One hundred microliters of anti-αGST IgG conjugated to horseradish peroxidase were added to each well and incubated at room temperature for 60 min with uniform shaking. Wells were washed six times, after which 100 μL of stabilized liquid TMB solution were added to each well and the plates incubated for exactly 15 min at room temperature. Reactions were stopped by the addition of 100 μL of 1 N sulfuric acid. The absorbance (optical density [OD]) was measured at 450-630 nm with a microplate reader (Molecular Devices SpectraMax M2^{e}).

3.2.2 πGST ELISA. Enzyme-linked immunosorbent assays were performed as described in the Biotrin Pi GST EIA kit protocol. First, plasma samples were diluted 1:5 with sample diluent (protein containing solution with added stabilizers). One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with IgG directed against πGST and the plates were incubated at room temperature (20-25^{o} C) for 60 min with uniform shaking. Plates were washed 4 times with wash solution (phosphate buffered saline with Tween 20). One hundred microliters of anti-πGST IgG/ horseradish peroxidase conjugate were added and incubated at room temperature for 60 min with uniform shaking. Wells were washed four times after which 100 μL of stabilized liquid TMB solution were added, and the plates were incubated for exactly 15 min at room temperature. Reactions were stopped by the addition of 100 μL 0.5 M sulfuric acid. The absorbance (optical density [OD]) was measured at 450-630 nm with a microplate reader (Molecular Devices SpectraMax $M2^e$).

3.2.3 Transferrin. Enzyme-linked immunosorbent assays were performed as described in the AssayMax Human Transferrin ELISA kit protocol. First, serum samples were diluted 1:2000 with MIX diluent A (containing 0.01% thimerosal). Twenty-five microliters of diluted sample were added to the wells of 96-well microtiter plates coated with polyclonal antibody against human transferrin. Immediately, 25 μL of biotinylated transferrin were added to each well and incubated for 60 min at 20-30^o C. Plates were washed 5 times with wash buffer (containing 0.01% thimerosal). Fifty microliters of streptavidin-peroxidase conjugate diluted in MIX diluent were added and incubated for 30 min at 20-30^o C. Wells were washed five times, 50 μL of stabilized peroxidase chromogen substrate (<0.05% *w/v* 3,3',5,5'-tetramethylbenzidine in H_2O) were added to each well, and plates were incubated for 10 min at 20-30^o C. Reactions were stopped by the addition of 50 μL of 0.5 N hydrochloric acid. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax $M2^e$).

3.2.4 Soluble Transferrin Receptor (sTfR). Enzyme-linked immunosorbent assays were performed as described in the BioVendor Human sTfR ELISA kit protocol. First, serum samples were diluted 1:50 with dilution buffer. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with mouse monoclonal anti-sTfR antibody and incubated for 60 min at 30-35^o C with uniform shaking at 300 rpm. Plates were washed 3 times with wash solution (containing 0.05% thimerosal). One hundred microliters of mouse monoclonal anti-sTfR antibody, horseradish peroxidase conjugate were added to all wells and incubated for 60 min at 30-35^o C with uniform shaking at 300 rpm. Wells were washed 3 times, 100 μL of TMB substrate solution were added, and plates were incubated 10 min at 20-30^o C in the dark. Reactions were stopped by the addition of 100 μL of 1.96% sulfuric acid. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax $M2^e$).

3.3 Blood Chemistry Analyses

3.3.1 Serum Iron and Total Iron-Binding Capacity (TIBC). Serum iron and TIBC were measured in 65 uL of serum using Dade Behring reagents on the Dimension® RxL Max® Integrated Chemistry System. Samples were thawed to room temperature prior to analysis.

3.3.2 Ferritin. Ferritin is commonly used as a clinical indicator of iron storage. Ferritin levels were measured in 25 uL of serum using ADVIA ReadyPack® reagents on the ADVIA Centaur® XP Immunoassay System. Samples were thawed to room temperature prior to analysis.

3.3.3 Percent Transferrin Saturation (%TS). %TS was calculated using the formula %TS = (Serum Iron / TIBC) x 100%.

4. RESULTS

4.1 Enzyme-Linked Immunosorbent Assay Testing

4.1.1 αGST ELISA. Serum from 48 male and 42 female subjects was analyzed for αGST concentration (**Figure 2**). Individual αGST concentrations ranged from 1531 to 84273 ng/L (mean = 9552 ± 9554 ng/L). There were no statistically significant differences ($p = 0.267$) between men (1531 to 84273 ng/L, mean = 10165 ± 12233 ng/L) and women (2123 to 27126 ng/L, mean = 8956 ± 5081 ng/L).

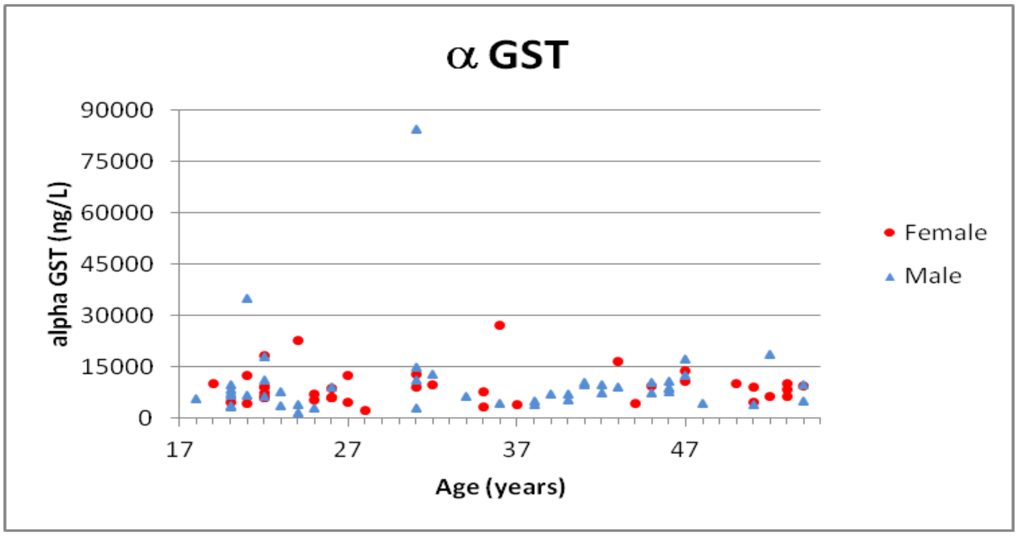

Figure 2. Age versus *α*GST concentration in sample cohort.

4.1.2 πGST ELISA. Plasma from 48 male and 42 female subjects was analyzed for πGST concentration (**Figure 3**). Individual πGST concentration ranged from 37 to 257 µg/L (mean = 122 ± 44 µg/L). There were no statistically significant differences in πGST ($p = 0.195$) between men (37 to 234 µg/L, mean = 117 ± 44 µg/L) and women (64 to 257 µg/L, mean = 125 ± 44 µg/L).

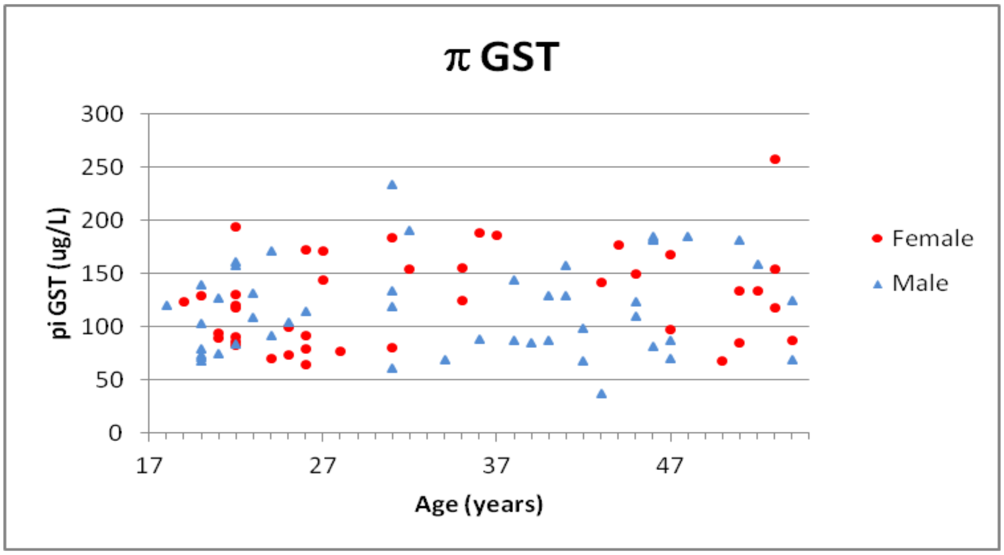

Figure 3. Age verses πGST concentration in sample cohort**.**

4.1.3 Transferrin ELISA. Serum from 48 male and 42 female subjects was analyzed for transferrin concentration (**Figure 4**). Individual transferrin concentration ranged from 1343 to 12468 ug/mL (mean = 5435 ± 2104 ug/mL). There were no statistically significant differences ($p = 0.226$) between men (1824 to 12468 ug/mL, mean = 5253 ± 2043 ug/mL) and women (1343 to 9994 ug/mL, mean = 5592 ± 2178 ug/mL).

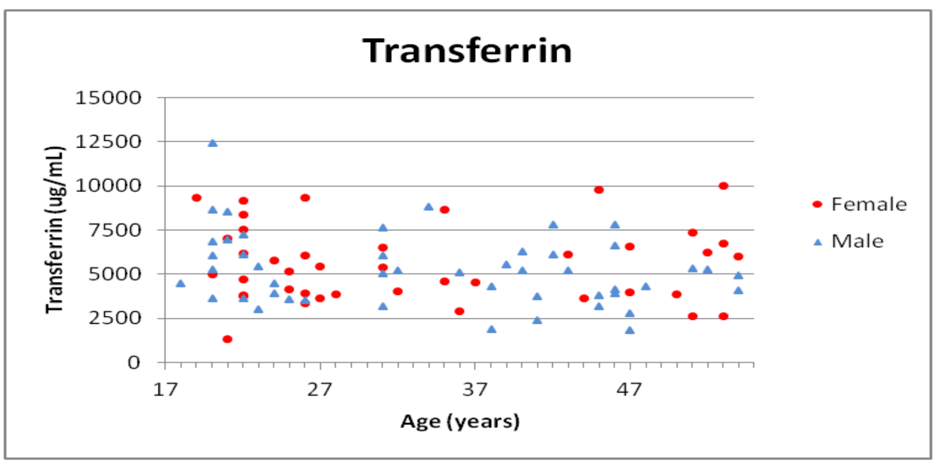

Figure 4. Age versus transferrin concentration in sample cohort.

4.1.4 Soluble Transferrin Receptor (sTfR) ELISA. Serum from 48 male and 42 female subjects was analyzed for sTfR concentration (**Figure 5**). Individual sTfR concentration ranged from 0.46 to 4.82 ug/mL (mean = 1.37 ± 0.68 ug/mL). There were no statistically significant differences (p = 0.341) between men (0.46 to 4.82 ug/mL, mean = 1.34 ± 0.75 ug/mL) and women (0.64 to 3.37 ug/mL, mean = 1.40 ± 0.61 ug/mL).

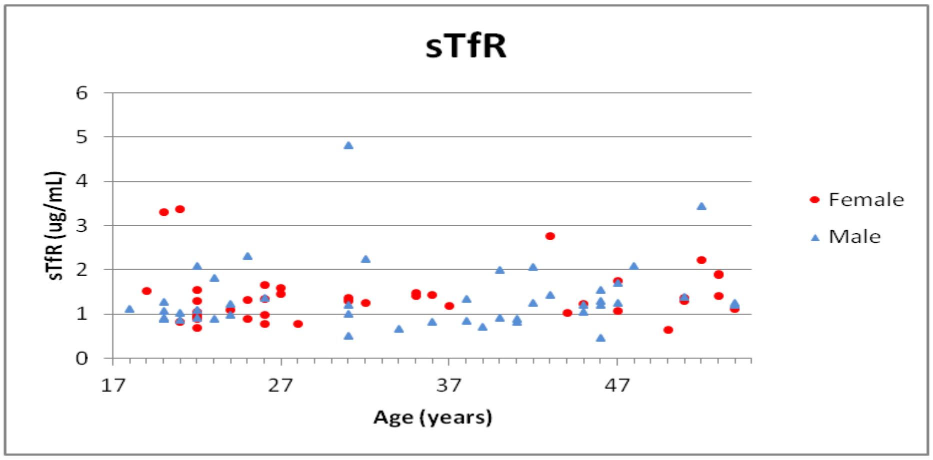

Figure 5. Age versus sTfR concentration in sample cohort.

4.2 Blood Chemistry Analyses

4.2.1 Serum Iron and Total Iron-Binding Capacity (TIBC). Serum from 48 male and 42 female subjects was analyzed for iron concentration (**Figure 6**). Individual serum iron concentration ranged from 20 to 278 mg/dL (mean = 103 ± 52 mg/dL). There were statistically significant differences (p = 0.00089) between men (20 to 278 mg/dL, mean = 119 ± 54 mg/dL) and women (26 to 270 mg/dL, mean = 85 ± 44 mg/dL).

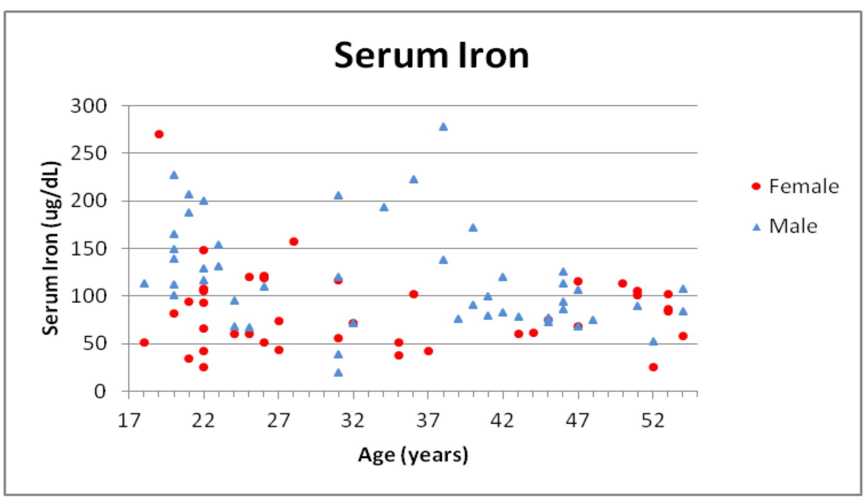

Figure 6. Age versus serum iron concentration in sample cohort.

Serum from 48 male and 42 female subjects was analyzed for total iron binding capacity (**Figure 7**). Individual TIBC concentration ranged from 217 to 634 ug/dL (mean = 389 ± 90 ug/dL). There were statistically significant differences ($p = 0.00132$) between men (217 to 568 mg/dL, mean = 363 ± 75 ug/dL) and women (258 to 634 ug/dL, mean = 419 ± 97 ug/dL).

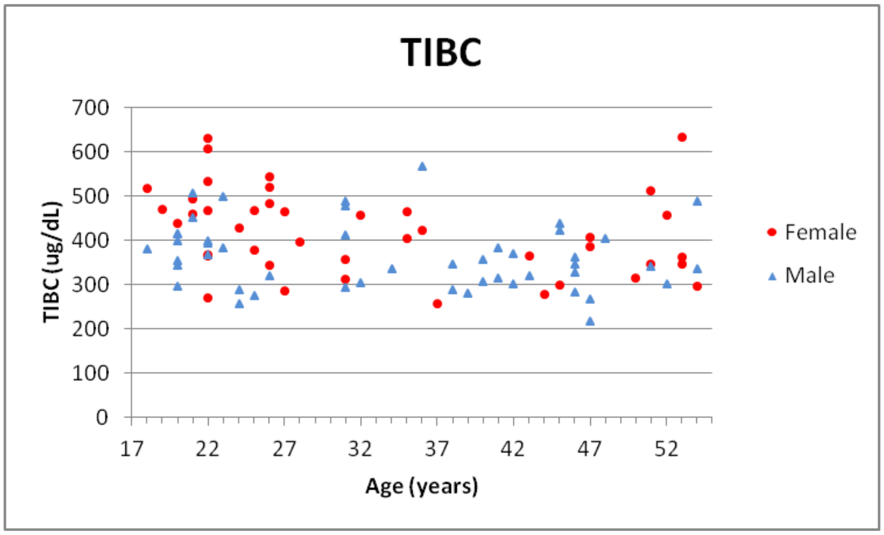

Figure 7. Age versus TIBC concentration in sample cohort.

4.2.2 Ferritin. Serum from 48 male and 42 female subjects was analyzed for ferritin concentration (**Figure 8**). Individual ferritin concentration ranged from 1.3 to 548.4 ng/mL (mean = 86.5 ± 89.3 ng/mL). There were statistically significant differences ($p = 0.0000133$) between men (10.9 to 548.4 ng/mL, mean = 86.5 ± 89.3 ng/mL) and women (1.3 to 191.5 ng/mL, mean = 46.8 ± 53.8 ng/mL).

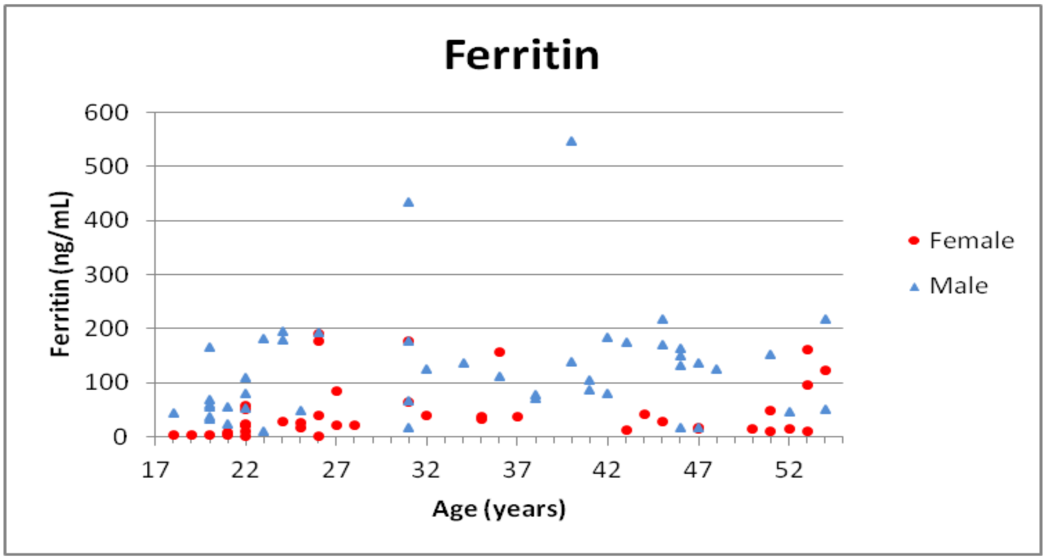

Figure 8. Age versus ferritin concentration in sample cohort.

4.2.3 Percent Transferrin Saturation (%TS). Individual transferring saturations ranged from 4.28 to 96.53% (mean %TS = 27.55 ± 14.38) (**Figure 9**). There were statistically significant differences ($p = 0.0000162$) between men (6.78 to 96.53%, mean %TS = 33.21 ± 15.08) and women (4.28 to 57.57%, mean %TS = 21.08 ± 10.39).

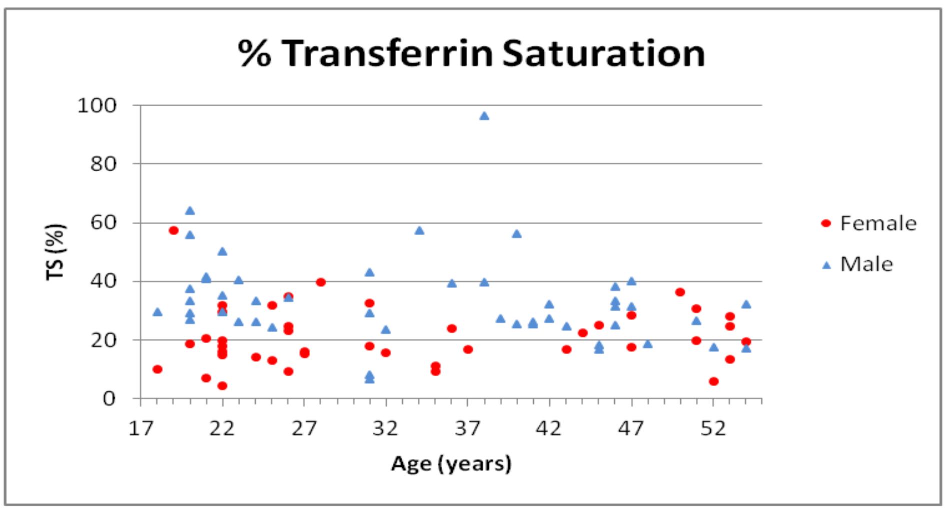

Figure 9. Age versus % transferrin saturation (%TS) in sample cohort.

4.3 Regression Analysis of Ungrouped Data

All data were the average of duplicate determinations. Analyses were completed using Minitab 15 Statistical Software™ from Minitab Inc and MATLAB® Statistics Toolbox™ from The

MathWorks, Inc. Regression analyses were performed using either αGST or πGST concentration as response variable and age, gender, transferrin, sTfR, iron, TIBC and %TS as indicator variables. Analyses required transformation of the response variables to satisfy the assumptions of normality and equal variance.

4.3.1 αGST Data. Regression analysis was performed with serum αGST concentrations as the response variable with age, gender, transferrin, sTfR, iron TIBC, %ISAT, and ferritin as the predictors. The analysis required a transformation of the response variable in order to satisfy assumptions of normality and equal variance (Tables 2-4). After transformation of the response variable, the parameters of age, sTfR, iron and %TS were identified as significant predictors. The R^2 value for the regression model was a very low 25.5%. A positive association was identified between age and αGST concentration, indicating that increased age is associated with higher αGST concentrations in serum (**Figure 10**). Negative associations were identified between transformed αGST concentration and serum iron and (**Figure 11**) as well as %TS (**Figure 12**). However, in all four cases the correlation is very weak. Unlike the linear associations observed with age, serum iron and %TS as indicator variables, an increase in transformed αGST concentration values was only observable using a quadratic regression model with sTfR as an indicator variable (**Figure 13**).

Table 2. αGST Regression Plot Parameters: Summary of Fit.

Summary of Fit	
RSquare	0.255572
RSquare Adjusted	0.192023
Root Mean Square Error	4076.842
Mean of Response	192214.3
Observations (or Sum Weights)	90

Table 3. αGST Regression Plot Parameters: Analysis of Variance.

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	467898812	66842687	4.0217
Error	82	1362892527	16620641	Prob > **F**
C. Total	89	1830791339		00.0008*

Table 4. αGST Regression Plot Parameters: Parameter Estimates.

Parameter Estimates				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob > |t|**
Intercept	189732.61	2438.203	77.82	<0001*
Age	76.891162	40.82519	1.88	0.0632
sTfR	-472.39	1091.17	-0.43	0.6662
(sTfR-1.3534)*(sRfR-1.36534)	1173.0051	491.3059	2.39	0.0193*
Iron	31.164953	22.04088	-3.06	0.0030*
(Age-33.7889)* (Iron-103.411)	0.0020047	0.001101	1.82	0.0720
Age-33.7889)* (%ISAT-0.27548)	1821.993	702.5931	2.59	0.0113*

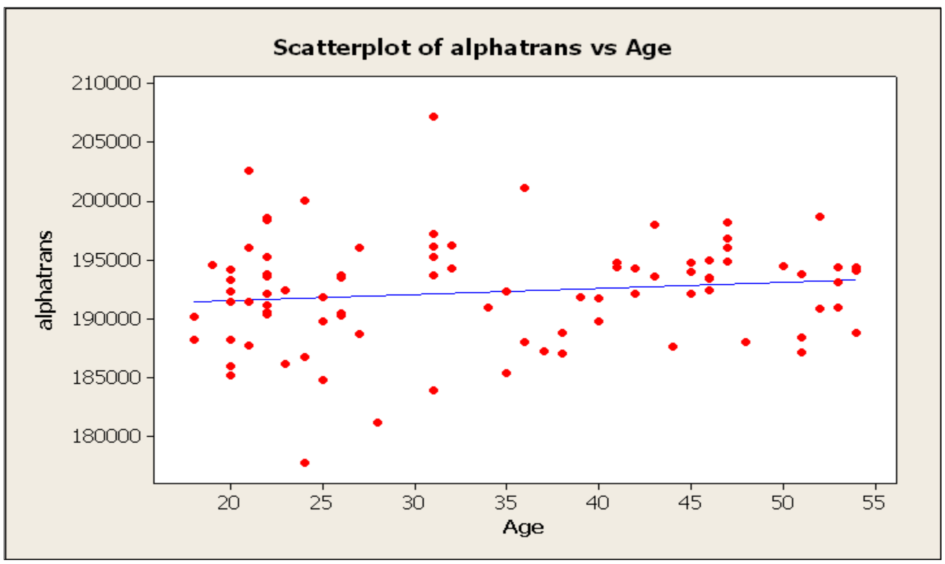

Figure 10. Age versus Transformed αGST concentration values (alphatrans).

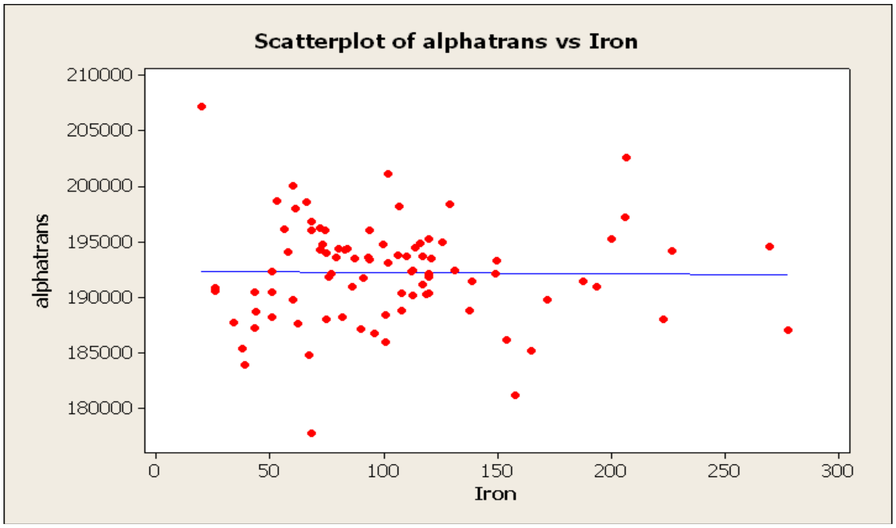

Figure 11. Serum iron versus Transformed αGST concentration values (alphatrans).

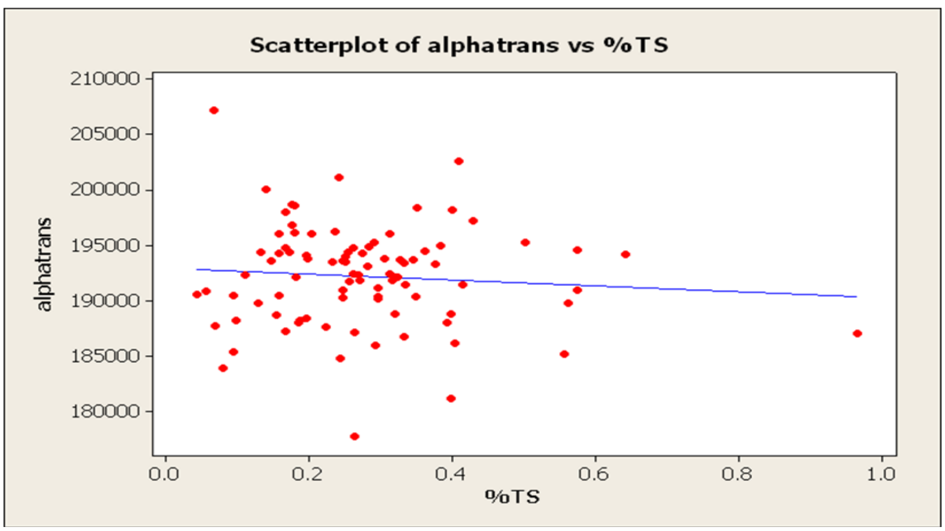

Figure 12. %TS versus Transformed αGST Concentration values (alphatrans).

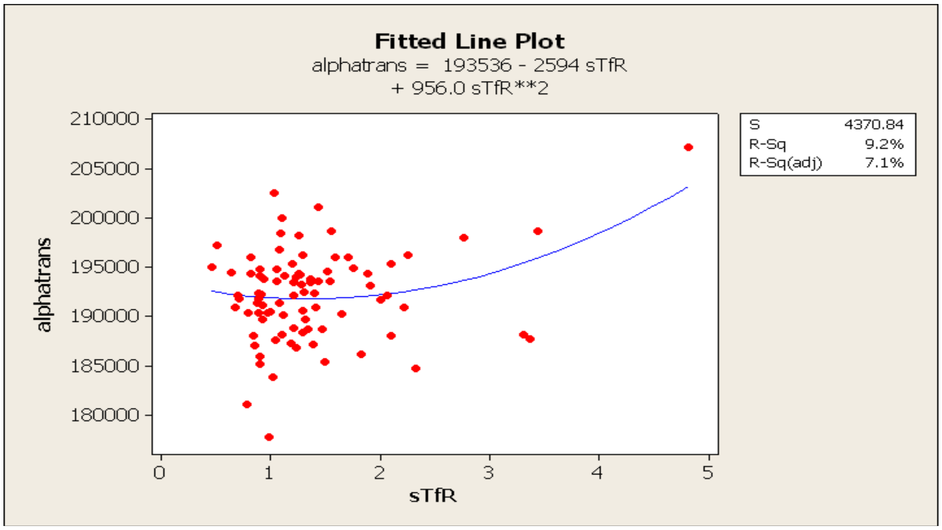

Figure 13. sTfR versus Transformed αGST Concentration values (alphatrans).

4.3.2 Ungrouped αGST Concentration Values: Interactions with Age and Iron. It was shown that the interactions between age and iron, and between age and %ISAT, were significant predictors of αGST concentrations in the serum. In order to create a pictorial representation of these interactions, age and iron were placed into three groups, numbered 1 to 3 in increasing order against transformed αGST values (alphatrans) (**Figure 14**). In this graph, boxes indicate interquartile ranges as well as the medians (horizontal line inside box). Outliers are indicated as dots. In Figure 14, nested plots of αGST by age and iron can be seen. In the first two age groups, αGST is seen to peak at the middle concentration of iron (**Figures 14A** and **14B**). In the oldest age group, the αGST peaked at the lowest concentration of iron (**Figure 14C**). This trend was also seen using the same parameters against untransformed αGST values (**Figure 15**).

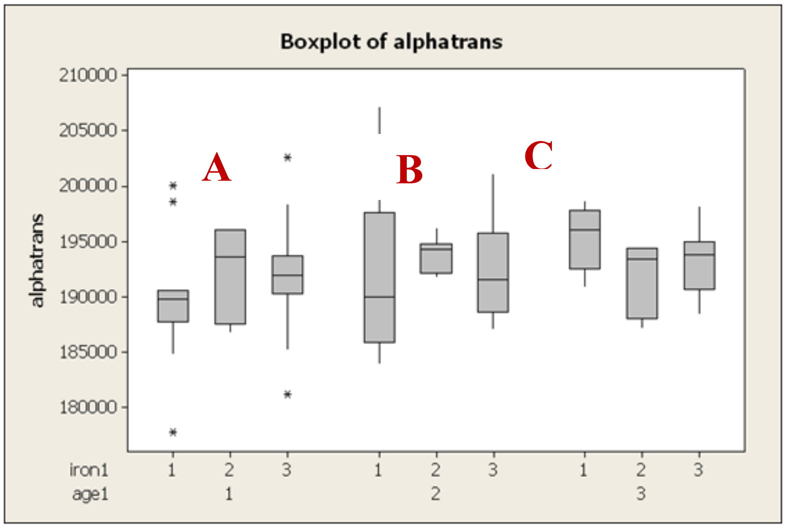

Figure 14. Box plot of Transformed αGST (alphatrans) with age and iron.

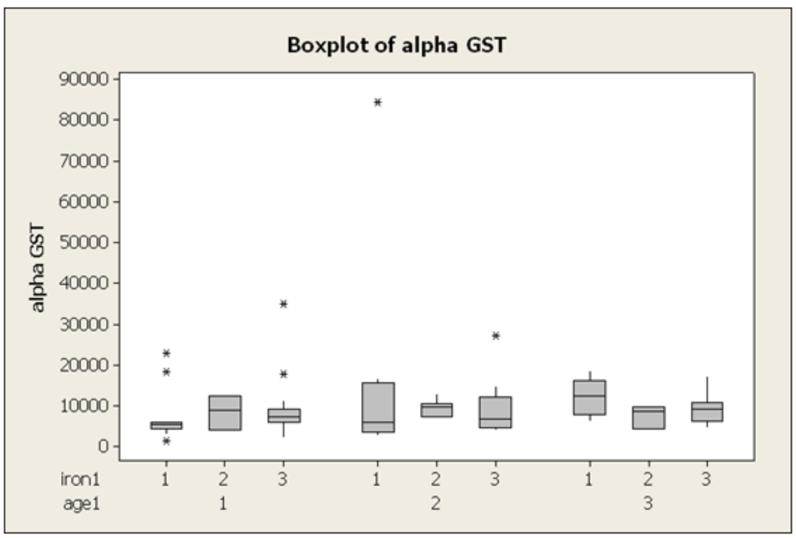

Figure 15. Boxplot of αGST with age and iron.

4.3.3 Ungrouped αGST Concentration Values: Interactions with Age and %ISAT. A similar examination of the interactions of transformed αGST with age and %ISAT indicate that αGST levels increased with %ISAT within the first and third age groups (**Figure 16**). In the middle age group, αGST peaked at the middle level of %ISAT. The same results were also seen using a boxplot analysis of **un**transformed αGST with age and %ISAT (data not shown).

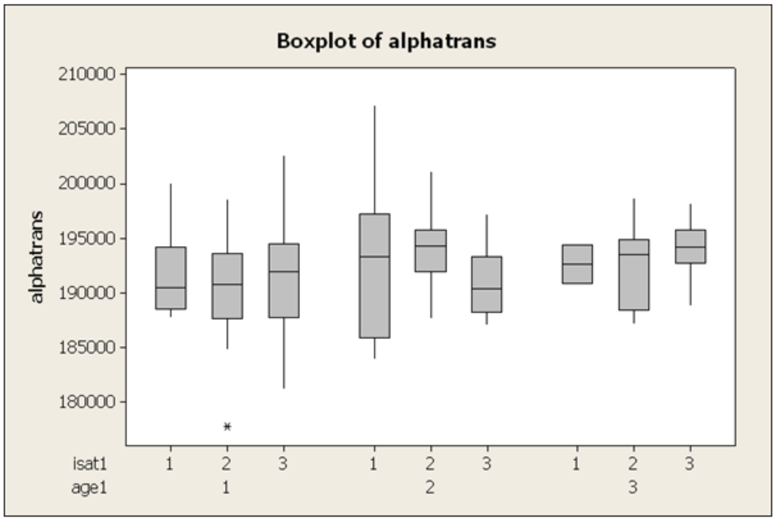

Figure 16. Boxplot of transformed αGST (alphatrans) with age and %ISAT.

4.3.4 Correlations with Ungrouped Plasma πGST Concentrations. Similar regression analyses were completed for the πGST data. The analyses required transformation of the response variable (πGST concentration) to satisfy the regression assumptions. Using regression analysis for transformed πGST values resulted in a quadratic model with serum iron levels identified as the only significant predictor of πGST concentration (**Figure 13**, **Tables 5-7**). In order to obtain this quadratic model, the significance was relaxed to $\alpha = .01$. However, the R^2 for the developed πGST model was only 6.9%.

Table 5. πGST Regression Plot Parameters: Summary of Fit.

Summary of Fit	
RSquare	0.06971
RSquare Adjused	0.048324
Root Mean Square Error	41.06229
Mean of Response	245.0576
Observations (or Sum Wgts)	90

Table 6. πGST Regression Plot Parameters: Analysis of Variance

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	2	10992.17	5496.09	3.2596
Error	87	146691.73	1686.11	Prob > **F**
C. Total	89	157683.91		0.0431*

Table 7. πGST Regression Plot Parameters: Parameter Estimates.

Parameter Estimates				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob > |t|**
Intercept	267.62111	10.41809	25.69	.0001*
Iron	-0.270286	0.106658	-2.53	0.0131*
(Iron-103.411)* (Iron-103.411)	0.0020047	0.001101	1.82	0.0720

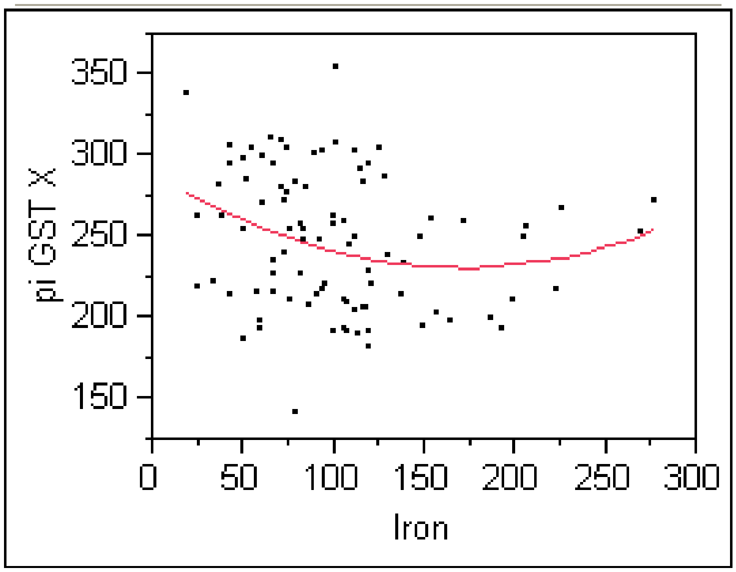

Figure 17. Regression plot of serum iron versus Transformed πGST Concentration values (pi GST X).

4.3.5 Ungrouped πGST Data: Other Modeling Efforts. Multiple iterations of model fitting were run in an attempt to establish associations between plasma πGST concentrations and the remaining indicator variables; however, no statistically significant relationships were identified. While the correlation of sTfR concentration with πGST concentrations were determined to be significant prior to transformation, no such significance was observed applying either linear or quadratic models with transformed πGST concentration values.

4.4 Multiple Linear Regression Analysis of Binned Data.

4.4.1 Binning Data into Normal and Elevated. Data were separated into two groups, elevated and normal, for all parameters studied. Published clinical data on normal and elevated concentrations were used to select the cutoff values to delineate between the two groups (**Table 8**). Analyses were completed using MATLAB® Statistics Toolbox™ from

MathWorks, Inc. Regression analyses were performed using either αGST or πGST concentration as response variable and age, gender, transferrin, sTfR, iron, TIBC and %TS as indicator variables. The indicator variables were close to normally-distributed, so no transformation was required. All data were the average of duplicate determinations.

Table 8. Cutoff Levels Used for Binning. Concentrations above these levels were binned into Elevated.

Parameter	Elevated Levels
αGST	> 10 μg/L
πGST	> 100 μg/L (plasma)
sTfR	>1.5 μg/mL
Transferrin	>3600 μg/mL
Iron	> 175 μg/L
TBIC	>450 μg/dL
%ISAT	>50% (males) >45% (females)
Ferritin	>250 ng/mL (male) >160 ng/mL (female)

4.4.2 R^2 and Regression Coefficients of Binned Data. The R^2 values were generated using multiple linear regression of the binned data. In examining αGST values as the response variable, two sets generated an $R^2 > 0.8$ (αGST vs. normal transferrin; αGST vs. normal ferritin), indicating that in these models over 80% of the variance could be accounted for by the indicator parameters (**Table 9**). R^2 values for αGST regression models were seen to be higher overall than those seen in πGST using similar modeling techniques (**Table 10**). However, both αGST and πGST statistical models indicate significant associations with GST, normal transferrin, elevated sTfR, and elevated iron.

The regression coefficient values represent a change in the response variable given a 1 unit change in the stated predictor parameter. Interestingly, regression coefficient values from αGST analyses indicate a greater sensitivity in sTfR and iron than those seen in πGST statistical models.

Table 9. R^2 and Correlation Coefficients using αGST Concentrations as the predictor.

	R^2	αGST Regression coefficients						
		Age	sTfR	Iron	IBCT	Ferritin	ISAT	Transferrin
αGST vs. elevated αGST	0.7361	-0.22025	4.034467615	0.577892	-0.16586	0.071929	-2.42517	-0.00109
αGST vs. elevated ferritin	Too few variables							
αGST vs. elevated IBCT	0.1934	-0.0374	-0.644319432	0.118352	-0.02208	-0.00162	-0.42234	0.000352
αGST vs. elevated iron	0.7734	-2.4373	-7.513862534	-0.88889	0.507174	0.217527	3.685244	0.003738
αGST vs. elevated ISAT	Rank deficient - predictor variables are not independent probably due to too few samples.							
αGST vs. elevated πGST	0.5301	-101.915	7977.602985	250.6775	-27.9586	47.15244	-885.503	-0.45054
αGST vs. elevated sTfR	0.6895	69.73334	8269.712198	369.777	-98.1864	67.22313	-1634.41	0.367387
αGST vs. elevated transferrin	0.1168	0.028524	0.828658745	0.116978	-0.01914	N/A	-0.41337	0.000126
αGST vs. normal αGST	0.1511	0.063258	-0.530135475	0.022495	0.000581	-0.00024	-0.07461	0.000107
αGST vs. normal ferritin	0.0816	0.049496	0.651131291	0.11084	-0.01736	-0.00339	-0.38415	-0.00016
αGST vs. normal IBCT	0.4601	-0.0882	7.288708621	0.031478	-0.01167	0.032575	-0.21583	-0.00067
αGSt vs. normal iron	0.4295	-0.05367	5.2660233	0.150148	-0.03615	0.03645	-0.77781	-0.00059
αGST vs. normal ISAT	0.3924	-0.05288	5.129834215	0.257796	-0.0514	0.042947	-1.12295	-0.00023
αGST vs. normal πGST	0.1245	70.05476	-340.7989315	12.69368	-9.32598	-5.31989	-119.847	-0.15562
αGST vs. normal sTfR	0.1191	14.9016	3771.840539	127.8873	-16.3882	0.680121	-416.988	-0.0799
αGST vs. normal transferrin	0.8914	-0.33987	12.84481277	0.130527	0.00693	0.106365	-0.00686	-0.01082

Table 10. R^2 and Correlation Coefficients using πGST concentration as the predictor.

	R^2	πGST Regression coefficients						
		Age	sTfR	Iron	IBCT	Ferritin	ISAT	Transferrin
πGST vs. elevated αGST	0.4387	-0.39104	-2.22881228	3.151102	-1.00508	0.081409	-13.4464	-0.00508
πGST vs. elevated ferritin	Too few variables							
πGST vs. elevated IBCT	0.1669	0.416644	-8.511734572	0.791773	-0.28428	-0.09969	-4.26459	0.003143
πGST vs. elevated iron	0.7409	-3.98427	-28.28382823	-0.09218	0.432	0.268939	3.243887	-0.00034
πGST vs. elevated ISAT	Rank deficient - predictor variables are not independent probably due to too few samples							
πGST vs. elevated πGST	0.3364	0.360959	4.680361696	0.107789	-0.17711	0.022297	-0.82216	-0.00126
πGST vs. elevated sTfR	0.5762	0.680597	-4.854234531	3.595405	-0.68847	0.006954	-16.1723	-0.00997
πGST vs. elevated transferrin	0.1009	0.337077	6.514631029	0.021304	-0.07948	N/A	-0.47716	-0.00273
πGST vs. normal αGST	0.1475	0.783888	-3.009125144	-0.35732	0.003646	-0.02965	0.764065	-0.00393
πGST vs. normal ferritin	0.0898	0.479301	5.824376335	0.220123	-0.10381	-0.04035	-1.19933	-0.00266
πGST vs. normal IBCT	0.1678	0.175588	13.35431035	-0.0998	-0.03999	0.042648	-0.29357	-0.00482
πGST vs. normal iron	0.1745	0.361015	6.267600779	0.678581	-0.22394	0.066079	-3.8235	-0.00188
πGST vs. normal ISAT	0.183	0.379139	6.040854725	0.928046	-0.28543	0.037831	-5.00855	-0.00191
πGST vs. normal πGST	0.0541	0.22978	4.312140608	0.154746	-0.01867	-0.03007	-0.62846	-0.00102
πGST vs. normal sTfR	0.0547	0.408309	-10.26627365	-0.08468	-0.04275	-0.00209	-0.06139	-0.00162
πGST vs. normal transferrin	0.6147	0.983792	5.755937998	6.737295	-1.51798	0.251232	-21.2758	-0.01043

5. CONCLUSIONS

The purpose of the original study was to develop a human model of sub-clinical hepatic damage to be used for pre-validation of in-house biomarker discoveries. In the course of reviewing existing literature on the association of GST and iron components in support of our HFE model, we discovered a lack of relative data on the range of GST levels and associations with iron and related iron parameters. In theory, abnormally high concentrations of cellular iron should result in some level of liver damage which in turn should be signaled by elevated GST levels in the blood. However, for accurate model development it was necessary to examine other factors which impact baseline levels of GST (such as age and gender) as well determine the exact nature of iron status as correlated with α and π GST levels in the blood. Matched human serum/plasma samples were analyzed for GSTs and iron parameter concentrations to provide additional data in fine tuning this potential testing model for future use in biomarker validation, especially needed for validation of markers to uncharacterized/mixed toxin exposures (original data presented in **Appendix A**).

In the original study, individuals with an elevated %TS did have elevated liver enzyme activity, with the exception of αGST and alanine aminotransferase (ALT). In this report, significant correlations with iron and iron components were seen with α and π GST levels. In addition, a basic understanding of the confounding factors of age and gender of GST biomarker levels was established.

5.1 Ungrouped Data

5.1.1 Iron Parameter Sample Analyses. The iron parameter concentrations in the collected cohort samples were examined for gender differences. Measurements of serum transferrin and sTfR levels indicated no statistically significant differences between male/female samples. However, significant gender differences were seen in the cohort sample set in serum levels of iron, TIBC, ferritin, and %TS.

5.1.2 GST Sample Analyses. Examination of both αGST and πGST demonstrated no statistically significant differences between male and female samples when analyzed from serum and plasma, respectively.

5.1.3 Regression Analysis of Data Sets. Regression analysis with ungrouped data using αGST as a response variable identified the interaction between age and iron as well as between age and %ISAT as significant predictors of αGST (**Figure 16**). This statement is more relevant than stating that αGST increases with age and iron but decreases with %ISAT. Plots of untransformed αGST data indicated the same trends.

Similar analyses for πGST resulted in a quadratic model with only iron as a significant predictor (**Figure 17**). In this model, a transformation of the response variable was required to satisfy the regression assumptions. In order to get this model, the significance was relaxed to $\alpha = 0.10$, giving The output for this model showed that, using transformed πGST data, the R^2 for the quadratic model was only a very low 6.9%.

5.2 Binned Data

When the data sets have been binned into 'Elevated' versus 'Normal' groupings based on current clinical delineations, higher R^2 values and stronger significance is seen using αGST as a response variable (**Table 9**). Using binned data, elevated levels of iron and sTfR are strongly significant predictors of elevated αGST levels (0.7734 and 0.6895, respectively), but not normal levels. Circulating sTfR has been seen to increase with iron requirements or cellular growth.[27] and is reflective of the status of tissue iron stores. The sTfR is elevated under conditions of iron deficiency. Therefore increased sTfR may be indicative of a physiological injury reflecting a need for increased cellular iron, and the concomitant increase in αGST may be reflective of primary or secondary injury. No medical data was collected on the cohort samples, therefore no conclusions can be drawn regarding associations with physiological conditions.

It has been seen that transcriptional induction of a GST isoform upon iron overload is thought to be a consequence of the production of ROS via an antioxidant responsive element (ARE) in the promoter region of the gene. Due to limited data for binned elevated ferritin, no definitive conclusion could be made in regards to its correlation with GST expression. Published data indicates that use of ferritin levels may be of limited value in determining iron status, especially in chronic disease states, unless different cutoff values are used.[28] Therefore, a more thorough examination of the correlation of αGST and ferritin would require information on subject health for more accurate binning of the ferritin data.

Unlike the ungrouped data analyses, statistically significant associations can be seen with πGST albeit not as stringent (**Table 10**). Analysis also indicates that both α and π GST levels associate with normal transferrin concentrations in the blood. However, significant association is lost for both GST variants when analyzed against elevated transferrin.

The data presented here does not support a relationship between αGST and πGST levels as the R^2 values do not indicate correlation of the two GST variant concentrations in the blood.

6. REFERENCES

[1]Eaton DL, Bammler TK. "Concise Review of the Glutathione S-Transferases and their Significance to Toxicology." (1999) *Tox Sci* **29**:156-164.

[2]Salinas AE, Wong MG. "Glutathione S-transferases – a review." *Curr Med Chem* (1999) **6**:279-309.

[3]Hubatsch I, Ridderstrom M, and Mannervik B. "Human glutathione tansferase A4-4: An Alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxication. (1998) *Biochem. J.* **330**:174-179.

[4]Townsend DM, Tew KD. "The role of glutathione-S-transferase in anti-cancer drug resistance." *Oncogene* (2003) **22**:7369-7375.

[5]Sheehan D, Meade G, Foley VM, and Dowd CA. "Structure, function, and evolution of glutathione transfereases:implications for classification of non-mammalian members of an ancient enzyme superfamily." *Biochem. J*. (2001) **360**:1-16.

[6]Boyer TD. "The glutathione S-transferases: an update". (1989) *Hepatology* **9**:486–496

[7]Hiley C, Fryer A, Bell J, Hume R, Strange RC. The human glutathione *S*-transferases: immunohistochemical studies of the developmental expression of alpha- and pi-class isoenzymes in liver. *Biochem J.* (1988) **254**:255-259.

[8]Federico A, Tuccillo C, Crafa E, Loguercio C. "The significance of alpha-glutathione S-transferase determination in patients with chronic liver diseases." *Minerva Gastroenterol Dietol* (1999) **45**:181-185.

[9]Gunshin H, Mackenzie B, Berger UV, et al. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* (1997) **388**:482-488.

[10]Fleming MD, Trenor CC III, Su MA, et al. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat Genet* (1997) **16**:383-386.

[11]Ponka P, Beaumont C, Richardson DR. "Function and regulation of transferrin and ferritin." *Semin Hematol.* (1998) **35**:35-54.

[12]Iancu TC. "Ultrastructural aspects of iron storage, transport, and metabolism." *J Neural Transm* (2011) **118**:320-335.

[13]Pedersen JZ, De Maria F, Turella P, Federici G, Mattei M, Fabrini R, Dawood KF, Massimi M, Caccuri AM, Ricci G." Glutathione transferases sequester toxic dinitrosyl-iron complexes in cells. A protection mechanism against excess nitric oxide." *J Biol Chem*. (2007) **282**:6364-6371.

[14]Camaschella C and Poggiali E. "Inherited disorders of iron metabolism." *Curr Opin Pediatr*. (2011) **23**:14-20.

[15]Darshan D, Frazer DM, Anderson GJ. "Molecular basis of iron-loading disorders." *Expert Rev Mol Med.* (2010) **12**:e36.

[16] Hanson EH, Imperatore G, Burke W. "HFE gene and hereditary hemochromatosis: a HuGE review." *Am J Epidemiol. (*2001) **154**:193-206

[17] Worwood M. “HFE Mutations as risk factors in disease.” *Best Pract Res Clin Haematol*. (2002) **15**:295-314.

[18]Muñoz M, García-Erce JA, Remacha AF. “Disorders of iron metabolism. Part II: iron deficiency and iron overload.” *J Clin Pathol*. (2011) **64**:287-296.

[19]Sharma V, Kumar B, and Saxena R. “Glutathione S-transferase gene deletions and their effete on iron status in HbE/B thalassemia patients.” *Ann Hematol* (2010) **89**:411-414

[20]Tsay J, Yand Z, Ross PF, Cunningham-Rundles S, Lin H, Coleman R, Mayer-Kuckuk P, Doty SB, Grady RW, Giardina PJ, Boskey AL, Vogiatzi MG. “Bone loss caused by iron overload in a murine model: importance of oxidative stress.” Abstract from the 31st annual meeting of the American Society of Bone and Mineral Research, Denver, CO, September 11, 2009.

[21]Kohgo Y, Ijuta K, Ohtake T, Torimoto Y, Kato J. “Body iron metabolism and pathophysiology of iron overload.” *Int J Hematol*. (2008) **88**:7–15.

[22] Hershko C. “Pathogenesis and management of iron toxicity in thalassemia.” *Ann N Y Acad Sci* (2010) **1202**:1-9.

[23]Johnson N, Todd DM, Mauzy CA, Boyer JA, Minnick TA. “Examination of HFE C282Y/H63D Heterozygotes as a Potential Human Modeling System for Low Level Liver Damage.” (March 2006) Air Force Research Laboratory Technical Report AFRL-HE-WP-TR-2006-0021.

[24]Kowdley KV, Tait JF, Bennett, RL, Motulsky AG. “HFE-associated Hereditary Hemochromatosis.” In: Gene Reviews. Pagon RA, Bird TD, Dolan CR, Stephens K, editors. Seattle (WA): University of Washington, Seattle; 1993-.2000 Apr 03

[25]Sikorska K, Romanowski T, Stalke P, Jaskiewicz K, Bielawski KP. “Coexistence of HFE and rare UGT1A1 genes mutations in patients with iron overload related liver injury.” *Adv Med Sci.* (2010) **55**:108-110.

[26]DelRaso N, Mauzy C, Reitcheck R. “Biomarkers of Exposure to Toxic Substances. Volume I: Global Experimental Design: Biomarker Discovery for Early Prediction of Organ-Selective Toxicity.” (May 2009) Air Force Research Laboratory Technical Report Number AFRL-RH-WP-TR-2009-0102.

[27] R'zik S, Beguin Y. “Serum soluble transferrin receptor concentration is an accurate estimate of the mass of tissue receptors.” *Exp Hematol*. (2001) **29**:677-685.

[28]Lee EJ, Oh E-J, Park Y-J, Lee HK, and Kim BK. “Soluble Transferrin Receptor (sTfR), Ferritin, and sTfR/Log Ferritin Index in Anemic Patients with Nonhematologic Malignancy and Chronic Inflammation.” *Clin Chem* (2002) **48**:1118-1121.

7. LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

%ISAT	percent iron saturation
ROS	reactive oxygen species
HFE	hemochromatosis
GST	glutathione-S-transferase
πGST	pi glutathione-S-transferase
αGST	alpha glutathione-S-transferase
sTfR	soluble transferrin receptor
ELISA	enzyme-linked immunosorbant assay
EIA	enzyme immunoassay
OD	optical density
N	Normality
M	Molar concentration
TIBC	total iron-binding capacity
%TS	percent transferrin saturation
Wgts	weights
uL	microliter(s)
mL	milliliter(s)

8. APPENDICE A: COHORT DATA

Sample number	Age	Gender	αGST	πGST	Transferrin	sTfR	Iron	TIBC	%ISAT	Ferritin
Units	(years)		(ng/L)	(mg/L)	(mg/mL)	(mg/mL)	(mg/dL)	(mg/dL)	Iron/TIBC	(ng/dL)
1	51	**F**	4650.4	133.51	7359.377	1.294	101	513	19.69%	10.2
2	54	**M**	9850.3	124.58	4116.218	1.256	84	489	17.18%	50.9
3	36	**M**	4450.0	88.46	5094.696	0.836	223	568	39.26%	111.6
4	27	**F**	12435.7	143.88	3651.099	1.588	74	466	15.88%	85.0
5	35	**F**	3314.5	155.39	8667.215	1.49	38	404	9.41%	36.8
6	45	**F**	9455.6	149.61	9805.218	1.234	75	300	25.00%	29.3
7	31	**F**	8977.7	80.21	5366.211	1.357	117	358	32.68%	178.4
8	28	**F**	2122.6	76.98	3855.453	0.78	158	396	39.90%	21.3
9	43	**M**	8917.2	36.97	5196.056	1.433	79	320	24.69%	175.9
10	31	**M**	11258.8	61.14	5034.653	1.199	120	413	29.06%	177.0
11	35	**F**	7552.2	124.79	4620.636	1.402	51	464	10.99%	31.9
12	48	**M**	4473.9	184.88	4345.473	2.094	75	405	18.52%	125.9
13	37	**F**	4065.6	185.45	4556.746	1.183	43	258	16.67%	36.7
14	53	**F**	6342.1	154.03	6731.956	1.405	86	347	24.78%	95.2
15	24	**M**	3874.8	91.36	4503.392	1.231	96	288	33.33%	194.7
16	24	**M**	1530.9	170.88	3949.13	0.975	68	258	26.36%	179.0
17	26	**F**	5984.5	64.48	9323.175	0.991	51	545	9.36%	2.2
18	25	**M**	3096.4	104.66	3599.96	2.323	67	275	24.36%	48.5
19	31	**M**	2816.1	133.30	6069.445	1.013	39	489	7.98%	18.0
20	21	**F**	4329.2	93.65	7007.041	3.368	34	493	6.90%	4.0
21	24	**F**	22810.2	70.28	5776.7	1.098	60	427	14.05%	29.3
22	32	**M**	12905.7	190.35	5249.079	2.248	72	305	23.61%	124.4
23	31	**F**	12748.3	184.04	6545.747	1.295	56	311	18.01%	65.5
24	52	**F**	6290.0	133.69	6216.127	2.215	26	456	5.70%	15.1
25	20	**M**	3531.5	68.26	5285.57	0.898	101	345	29.28%	69.6
26	27	**F**	4802.9	171.72	5474.081	1.466	44	285	15.44%	21.7
27	46	**M**	7650.7	181.27	4132.431	1.296	113	361	31.30%	131.4
28	26	**F**	5902.3	171.9	3358.738	0.791	120	344	34.88%	39.0
29	51	**M**	4022.7	181.08	5344.261	1.382	90	341	26.39%	152.7
30	46	**M**	10761.9	184.89	3944.567	0.456	126	329	38.30%	164.1
31	54	**M**	4894.1	68.42	4935.952	1.202	108	337	32.05%	218.8
32	36	**F**	27126.4	188.60	2926.242	1.429	102	423	24.11%	158.1
33	22	**M**	17785.4	160.70	3662.471	1.089	129	367	35.15%	109.0
34	38	**M**	3979.3	144.14	1891.878	0.855	278	288	96.53%	78.1
35	22	**M**	11251.4	83.93	7247.631	2.091	200	398	50.25%	79.6

Sample number	Age	Gender	α GST	π GST	Transferrin	sTfR	Iron	TIBC	%ISAT	Ferritin
Units	(years)		(ng/L)	(mg/L)	(mg/mL)	(mg/mL)	(mg/dL)	(mg/dL)	Iron/TIBC	(ng/dL)
36	18	**F**	4535.253	176.06	7750.308	1.095	51	517	9.86%	4.3
37	20	**F**	4552.85	128.86	4982.38	3.309	82	439	18.68%	3.3
38	31	**M**	14871.911	119.42	7658.101	0.512	206	479	43.01%	67.1
39	22	**F**	9111.033	130.23	9187.192	0.932	106	533	19.89%	25.0
40	18	**M**	5740.209	119.51	4479.619	1.111	113	381	29.66%	45.1
41	20	**M**	8559.355	71.18	6077.5	1.278	150	399	37.59%	33.7
42	22	**F**	7329.698	119.96	3758.439	0.698	149	468	31.84%	56.9
43	21	**M**	6710.714	74.05	6984.819	0.878	188	453	41.50%	24.4
44	53	**F**	9945.528	117.86	9993.548	1.877	84	634	13.25%	9.7
45	20	**M**	6724.279	103.02	8647.55	1.079	139	415	33.49%	60.1
46	25	**F**	7088.018	99.28	5171.3	0.89	120	379	31.66%	27.3
47	20	**M**	3234.214	73.33	6876.747	0.895	165	296	55.74%	36.4
48	22	**F**	8919.644	118.18	7508.549	1.054	93	630	14.76%	10.7
49	22	**F**	6046.732	90.21	6157.433	1.292	26	607	4.28%	1.3
50	38	**M**	4860.962	86.75	4313.116	1.335	138	346	39.88%	70.9
51	40	**M**	6990.016	86.62	5235.917	2	91	356	25.56%	139.6
52	21	**M**	35039.773	126.43	8533.885	1.029	207	506	40.91%	55.0
53	50	**F**	10056.187	67.61	3878.369	0.642	114	315	36.19%	15.3
54	46	**M**	8841.081	81.38	6609.763	1.542	87	346	25.14%	18.3
55	22	**F**	5868.465	82.59	8368.142	0.967	108	365	29.59%	21.0
56	42	**M**	7376.247	67.99	6150.799	2.063	120	371	32.35%	183.3
57	32	**F**	9720.984	154.13	4045.487	1.26	72	456	15.79%	39.0
58	45	**M**	7395.383	123.83	3183.165	1.211	77	423	18.20%	171.0
59	47	**F**	10626.367	168.05	3975.582	1.757	116	408	28.43%	14.2
60	53	**F**	8343.258	257.26	2613.897	1.909	102	363	28.10%	162.1
61	22	**F**	18316.968	193.86	4737.35	1.548	66	367	17.98%	22.0
62	47	**M**	17187.647	70.28	2824.296	1.25	107	267	40.07%	135.8
63	41	**M**	9860.095	157.88	3762.174	0.816	80	315	25.40%	86.1
64	19	**F**	10153.525	123.37	9346.78	1.52	270	469	57.57%	4.2
65	26	**F**	5843.738	79.20	6065.644	1.65	119	483	24.64%	191.5
66	26	**M**	8996.359	114.42	3559.075	1.375	110	319	34.48%	192.3
67	45	**M**	10505.471	110.18	3816.41	1.051	73	438	16.67%	217.6
68	26	**F**	8776.702	92.06	3919.096	1.355	121	519	23.31%	177.9
69	21	**F**	12521.522	89.11	1342.847	0.817	94	460	20.43%	8.2
70	23	**M**	7617.456	108.47	3019.291	0.889	131	499	26.25%	10.9
71	41	**M**	10411.102	128.83	2427.578	0.902	100	383	26.11%	104.4
72	20	**M**	9638.724	139.10	3668.455	0.899	227	354	64.12%	55.8

Sample number	Age	Gender	α GST	π GST	Transferrin	sTfR	Iron	TIBC	%ISAT	Ferritin
Units	(years)		(ng/L)	(mg/L)	(mg/mL)	(mg/mL)	(mg/dL)	(mg/dL)	Iron/TIBC	(ng/dL)
73	51	**F**	9191.311	84.32	2622.87	1.364	106	347	30.55%	48.5
74	20	**M**	7516.914	78.89	12467.973	0.91	112	416	26.92%	166.3
75	23	**M**	3628.479	131.13	5474.494	1.821	154	382	40.31%	182.1
76	22	**M**	6513.543	157.19	6106.605	0.92	117	394	29.70%	53.6
77	43	**F**	16697.142	141.83	6139.577	2.766	61	365	16.71%	13.1
78	47	**F**	13974.136	97.87	6560.662	1.074	68	386	17.62%	16.3
79	34	**M**	6344.88	69.28	8842.979	0.671	194	337	57.57%	137.5
80	52	**M**	18499.399	158.80	5266.084	3.432	53	302	17.55%	47.1
81	46	**M**	8731.867	182.72	7829.328	1.207	94	282	33.33%	151.3
82	54	**F**	9541.21	87.53	6035.025	1.122	58	296	19.59%	122.3
83	47	**M**	12512.786	87.35	1823.885	1.709	68	217	31.34%	16.5
84	42	**M**	9786.145	98.19	7825.03	1.25	83	302	27.48%	79.6
85	39	**M**	7045.813	84.31	5576.537	0.706	76	280	27.14%	NA
86	44	**F**	4246.396	177.15	3668.335	1.037	62	277	22.38%	42.5
87	40	**M**	5475.159	128.95	6307.169	0.916	172	306	56.21%	548.4
88	31	**M**	84273.314	234.01	3202.919	4.818	20	295	6.78%	434.2
89	25	**F**	5467.978	73.06	4153.129	1.318	60	467	12.85%	17.9
90	22	**F**	5938.144	86.04	3795.758	0.89	43	271	15.87%	50.9